Uptake, Retention, and Excretion of Infectious Prions by Experimentally Exposed Earthworms

Sandra Pritzkow, Rodrigo Morales, Manuel Camacho, Claudio Soto

Prions are proteinaceous infectious agents that can be transmitted through various components of the environment, including soil particles. We found that earthworms exposed to prion-contaminated soil can bind, retain, and excrete prions, which remain highly infectious. Our results suggest that earthworms potentially contribute to prion disease spread in the environment.

Prions are unique infectious agents composed exclusively of a misfolded form of the prion protein (PrP^{Sc}) (*1*). Among prion diseases, chronic wasting disease, affecting cervids, and scrapie, affecting sheep, are highly contagious. Studies conducted in natural and experimental conditions suggest that these diseases likely are transmitted via environmental contamination and that soil is a primary vector (*2*–*4*). We examined whether earthworms contribute to environmental spread of infectious prions.

The Study

To investigate whether earthworms can act as carriers of infectious prions, we exposed groups of worms (*Eisenia fetida*) to soil previously mixed with brain homogenate (BH) from clinically diseased 263K Syrian golden hamsters (*Mesocricetus auratus*) (Harlan Envigo, https://www.envigo.com). For experiments, we homogenously mixed 375 g of Elliot soil (kindly provided by Joel Pedersen, Johns Hopkins University) with 25 mL of 10% wt/vol 263K brain homogenate. We assessed whether prions bind to worms or worm-associated soil by using protein misfolding cyclic amplification (PMCA) technology (*5*,*6*), which can detect prions down to the level of a single particle (*7*). Because PMCA efficiency can be severely affected by components in the inoculum (*6*), we first analyzed the effect of worm homogenate (WH) with or without soil on the efficiency of in vitro prion replication by PMCA (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/27/12/20-4236-App1.pdf). Our results indicated that whole WH does interfere with the reaction, but we could still obtain maximum amplification after 3 rounds of PMCA (Appendix Figure 1).

After verifying PMCA efficiency, we tested worms exposed to contaminated soil for different lengths of time. We collected worms from contaminated soil after 1, 3, 7, 14, and 28 days of exposure (Figure 1, panel A). PMCA results showed that worms exposed to prions take up PrP^{Sc} and efficiently sustain prion replication at all exposure times tested (Figure 1, panel B). We observed no PrP^{Sc} uptake in any worms exposed to control soil.

To study whether contaminated worms can transmit disease, we intraperitoneally injected hamsters with WH obtained from worms exposed to prion-soil mix for 28 days. To assess reproducibility, we used 3 different worms for this assay. Our results showed that worms exposed to prion-contaminated soil can transmit prion disease, albeit with variable efficiencies (Appendix Figure 2). Of the 3 worm extracts, 2 caused an attack rate of 4/5 and mean incubation periods of 237 (SE ±39) and 255 (SE ±25) days. A third WH transmitted disease to only 1/5 injected hamsters, which showed an incubation period of 272 days (Appendix Figure 2). For positive controls, we intraperitoneally injected groups of hamsters directly with 10% 263K BH. Terminal disease developed in all animals; the median incubation period was 151.4 (SE ±30) days (Appendix Figure 2). We confirmed prion disease by biochemical detection of protease-resistant PrP (Figure 1, panel C). We did not detect a PrP^{Sc} signal in hamsters that did not show clinical signs, suggesting the absence of preclinical prion disease in

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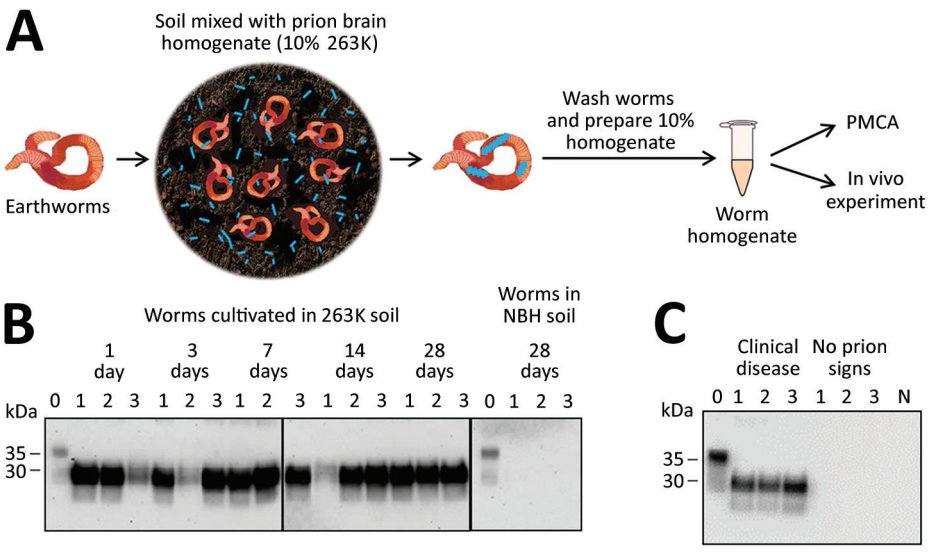

Figure 1. Detection of prion protein (PrP^{Sc}) attached to earthworms by PMCA and infectivity bioassay. A) Process for exposing earthworms to infected soil. Earthworms were placed in soil mixed with 10% wt/vol infected 263K hamster brain homogenate for 1, 3, 7, 14, or 28 days; worms were washed thoroughly, then prepared into a 10% homogenate for analysis. B) Results of PMCA on earthworms exposed to contaminated soil. As a control, earthworms also were exposed to soil mixed with NBH for 28 days and analyzed with the same methods. For each measurement, 3 worms were analyzed per time point in 3 different gels but blotted in the same membrane. Lane 0 is NBH used as a positive control for electrophoretic migration of the normal prion protein (PrP^{C}); lanes 1–3 indicate 3 different worms. Vertical lines between images depict membrane splicing. Numbers on the left indicate molecular weight markers. C) Biochemical analysis of brains of hamsters infected with worm homogenate. Groups of hamsters were injected with homogenates from 3 different worms exposed to prion contaminated soil; many of the animals developed prion disease (Appendix Figure 2, https://wwwnc.cdc.gov/EID/article/27/12/20-4236-App1.pdf). Brains were collected and homogenized and samples were digested with proteinase K (Sigma Aldrich, https://www.sigmaaldrich.com) at 50 μg/mL for 1 h at 37°C, except NBH (lane labeled N) used as a migration control. Numbers on the left indicate molecular weight markers. Results confirmed the presence of PrP^{Sc} accumulation in the brain of animals showing clinical signs of prion disease. NBH, normal hamster brain homogenate; PMCA, protein misfolding cyclic amplification.

those animals. Comparing incubation time and attack rate data obtained with WH and different dilutions of infected brain material suggests that the number of prions in each worm is equivalent to 1×10^{-5} to 1×10^{-6} dilution of infected brain. This estimation also is supported by analysis of the data by using a semiquantitative PMCA technique (*8*).

To investigate whether earthworms can retain infectious prions when exposed for different lengths of time to a prion-free environment, we exposed experimental subjects to prion-containing soil and subsequently transferred worms to naive soil (Figure 2, panel A). We collected worms from prion-containing soil after 7 days of exposure, thoroughly cleaned soil attached to the worms' surface, and cultivated worms in naive soil for another 1, 3, 7, 14, and 28 days; we collected and analyzed 4 worms at each time point. PMCA results showed PrP^{Sc}–positive signal for all 4 worms immediately after exposure to prion-contaminated soil (Figure 2, panel B). We found that 25%–50% of worms exposed to prion-free naive soil retained PMCA-detectable PrP^{Sc} (Figure 2, panel B). We observed no clear trend with the time of incubation in naive soil, and even animals exposed to prion-free soil for 28 days retained prions in their bodies (Figure 2, panel B).

To evaluate whether prion-contaminated earthworms excrete PrP^{Sc} back into the environment, we analyzed worm castings by using PMCA. We collected 2 worms exposed to prion-contaminated soil for 7 days and thoroughly washed worms with water. For casting collection, we placed animals in petri dishes and collected 8 pieces of casting from the petri dish to analyze PrP^{Sc} content by PMCA (Figure 2, panel C). The results showed 6/8 casting samples were positive for PrP^{Sc} (Figure 2, panel D). Of note, 3 samples had large amounts of PrP^{Sc} detectable by just 2 rounds of PMCA, indicating that earthworms exposed to prions in soil can take up and release PrP^{Sc} competent for prion replication.

Finally, to study whether some PrP^{Sc} molecules taken up from the soil remain attached to the body of the animal, we contaminated 6 worms by exposure to contaminated soil for 7 days. After washing to remove outside soil, we dissected animals to completely remove all soil particles inside the animal. We thoroughly washed worm bodies, homogenized them, and then used the homogenate for PrP^{Sc} detection by PMCA. Of the 6 six soil-void worms, 5 were positive for PrP^{Sc} after only 2 rounds of PMCA (Figure 2, panel E). The sixth worm became positive in the third PMCA round, as did control worms from which we

did not remove internal soil (Figure 2, panel E). These results suggest that a substantial part of PrP^{Sc} taken up by worms from soil remained attached to the body of the animal and not merely in the soil particles that the worm acquired.

Conclusions

The mechanisms implicated in the natural spread of infectious prions are not completely known. Some prion diseases, such as chronic wasting disease and scrapie, are thought to be highly transmissible through

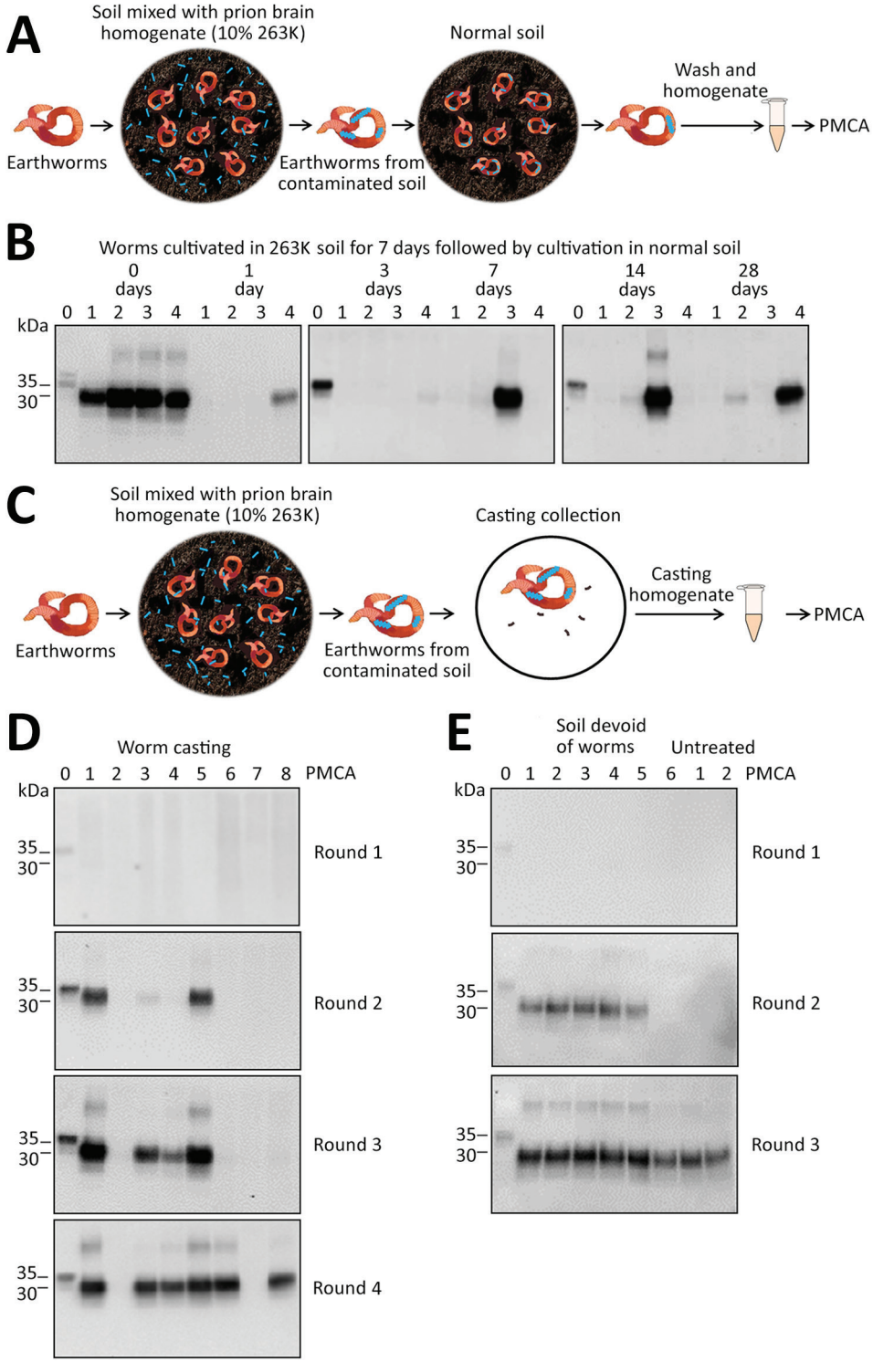

Figure 2. Detection of pathological prion protein (PrP^{Sc}) retention and dispersion by earthworms. A) Process for exposing earthworms to PrP^{Sc}–contaminated soil and analyzing for PrP^{Sc} retention. Worms were kept in PrP^{Sc}–contaminated soil for 7 days, then transferred to normal, prion-free soil and collected at various times. After collection, worms were thoroughly washed, homogenized, and used for PrP^{Sc} detection. B) Western blot analysis of PMCA of worm samples after cultivation in 263K-contaminated soil for 7 days and exposure to normal soil for 0, 1, 3, 7, 14, and 28 days. Lane 0 is normal brain homogenate (NBH) used as positive control; lanes 1–4 indicate 4 different worms for each time point. C) Process for collecting castings excreted by prion-contaminated worms to analyze for PrP^{Sc}. D) PMCA results for castings collected from earthworms exposed to 263K-soil for 7 days. Samples 1–8 were harvested and subjected to 4 PMCA rounds. E) Detection of PrP^{Sc} attached to 6 earthworms after exposure to prion-contaminated soil for 7 days. After collection and thorough washing, worms were dissected, and soil was carefully removed from the inside of the animal (soil-devoid worms). Worm carcasses were homogenized and used for PMCA detection of $PrPS^{c}$. As controls, we used 2 untreated worms, that is, worms for which no soil was removed. In panels B, D, and E, all samples were digested with proteinase K (Sigma Aldrich, https://www.sigmaaldrich.com) at 50 μg/mL for 1 h at 37°C, except the NBH used as a migration control of PrP^{C}. Numbers on the left indicate molecular weight markers. PMCA, protein misfolding cyclic amplification.

exposure to prion-contaminated environments (*2*,*3*). We previously demonstrated that infectious prions can attach to various components of the environment, including soil, plants, wood, and rock, and to several man-made surfaces, such as metals, plastic, and glass (*9*,*10*). However, little is known about how organisms living in the prion-exposed environment contribute to the spread of prions. In this study, we focused on earthworms (*E. fetida*) that live in close contact with known sources of prion infectivity in the environment, soil and diseased carcasses, and can move at a rate of 20–70 m/h (*11*,*12*). Our results demonstrate that earthworms can efficiently take up prions and act as vectors of prion disease transmission. In worms exposed to prion-contaminated soil, we noted PrP^{Sc} competent for both in vitro prion replication and in vivo infectivity. Even a relatively short exposure of 1 day was enough to contaminate all exposed worms. Of note, within 1 day after moving contaminated worms into prion-free soil, many earthworms were free of infectious particles. However, 25%–50% of worms retained PMCA-detectable PrP^{Sc} even 28 days after living in noncontaminated soil. Dissection of the worm's bodies to separate tissue from soil inside the animal showed that a substantial amount of PrP^{Sc} was in the worm bodies. Furthermore, analysis of the casting excreted by contaminated worms showed that 75% of the animal feces contained a relatively large quantity of PrP^{Sc} detectable by PMCA. These results suggest that earthworms exposed to prions remain potentially infectious for long periods and release prions back into the soil, therefore possibly contributing to the spread of infectious prions in nature.

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C.S. is inventor on several patents related to the protein misfolding cyclic amplification (PMCA) technology and is currently founder, chief scientific officer, and member of the board of directors of Amprion, Inc. (https://amprionme.com), a biotech company focusing on the commercial utilization of PMCA for prion diagnosis. R.M. is listed as an inventor in a patent associated with the PMCA technology. S.P. also has a conflict of interest related to the PMCA technology and Amprion, Inc. The University of Texas System has licensed intellectual property to Amprion, Inc.

About the Author

Dr. Pritzkow is an assistant professor in the Department of Neurology, University of Texas McGovern Medical School in Houston, Texas, USA. Her research interest is in the development of biochemical techniques for detecting prion-like neurodegenerative diseases.

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Appendix

Materials and Methods

Earthworm Exposure

We acquired Earthworms (*Eisenia fetida)* from Orcon Organic Control Incorporation (https://organiccontrol.com) and exposed worms to contaminated soil as described below. We obtained and homogenized brain tissue from 263K Syrian golden hamsters (*Mesocricetus auratus*) showing advanced stages of prion disease, as previously described (*1*). We homogenously mixed 375 g of Elliot soil (a gift from Joel Pedersen, John Hopkins University) with 25 mL of 10% wt/vol 263K hamster brain homogenate (BH) prepared in phosphate-buffered saline (PBS) plus complete protease inhibitor cocktail (Roche, https://www.roche.com).

We placed earthworms on contaminated soil and collected at the indicated times. After collection, we thoroughly washed earthworms with tap water to remove soil attached to the surface of the worm and prepared a 10% wt/vol whole worm homogenate (WH) in PBS using a Precellys 24 tissue homogenizer (Bertin Technologies, https://bertin-technologies.com). We stored homogenates at –80°C until used for in vitro or infectivity assays. In addition, we obtained worm casting from animals exposed to prion containing soil for 7 days. For casting collection, we placed worms in disposable petri dishes for 2 h and then collected released castings.

Serial Prion Replication

We prepared substrate for protein misfolding cyclic amplification (PMCA) reactions as previously reported (*2*). In brief, we intracardially perfused 3–4-week-old female Syrian golden hamsters with PBS supplemented with 5 mmol EDTA. We made a preparation of 10% wt/vol

normal brain homogenate (NBH) in conversion buffer (150 mmol NaCL and 1% Triton X-100 in PBS).

To detect prion protein (PrP^{Sc}), we added 13 µL of WH to 117 µL NBH and loaded the mixture into 0.2 mL PCR tubes (USA Scientific, https://www.usascientific.com), each of which contained three 0.24-cm diameter Teflon beads (Hoover Precision Products, http://www.hooverprecisionplastics.com). We placed the PMCA tubes in a Q700 microsonicator (Qsonica, https://www.sonicator.com) and submitted tubes to PMCA cycles of 29 m 40 sec incubation at 37°C and brief 20 sec sonication at ≈260 watts. After a round of 96 cycles, we transferred 10 µL of the amplified samples into 90 µL NBH and performed another PMCA round until the detection limit was reached. To control the PMCA reaction, we serially diluted 10% wt/vol BH from 263K prion-infected animals into the PMCA substrate. Negative controls included samples containing PMCA substrate alone.

Inhibitory Effect of Worm Homogenate on PMCA Assay

We spiked 10% wt/vol whole and soil-cleansed prion-free WH to serial dilutions of 263K BH to a final concentration of 10^{-4} to 10^{-9} and then used this to seed the PMCA. We diluted the spiked WH 10 times into NBH and PMCA reactions started as described above. We compared the amplification efficiency to a control PMCA of 263K spiked NBH.

Proteinase K Digestion Assay and Western Blot Test

We were able to detect PrP^{Sc} by incubating aliquots of the sample with 50 µg/mL proteinase K (PK; Sigma-Aldrich, https://www.sigmaaldrich.com) for 1 h at 37°C and shaking at 600 rpm in a ThermoMixer (Eppendorf, https://www.eppendorf.com). After incubation, we added SDS-sample buffer (Invitrogen, ThermoFisher Scientific, https://www.thermofisher.com) and 33 mmol Dithiothreitol (DTT; Sigma-Aldrich) and heated samples at 95°C for 10 min. PK-resistant prion protein was fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (ThermoFisher Scientific), transferred to a Hybond-ECL nitrocellulose membrane (Amersham GE Healthcare, https://www.gelifesciences.com) and detected by using 6D11 antibody (BioLegend, https://www.biolegend.com) diluted 1:5,000 in PBS/Tween. We visualized immunepositive bands by Amersham ECL Prime Western blotting detection kit (GE Healthcare) enhanced chemiluminescence assay (ECL) by using a ChemiDoc (Bio-Rad, http://www.bio-rad.com) image analysis system .

Infectivity Assay

Groups of 8–9-week-old female Syrian golden hamsters (Harlan Envigo, https://www.envigo.com) were intraperitoneally injected with 600 µL of 10% wt/vol homogenate of worms cultivated for 28 days in 263K contaminated soil. Homogenates from 3 different worms were used in this study. For negative controls, we used hamsters challenged with worms cultivated in NBH-treated soil. For positive controls, we directly injected hamsters interperitoneally with the 10% 263K BH. We pretreated positive and negative controls with ultraviolet radiation for 1 h to eliminate eventual bacterial contamination.

We monitored hamsters daily for signs of clinical disease. We measured progression of clinical signs by scoring the animals using a previously described system (*3*). We euthanized animals at advanced stages of prion disease, extracted brains and stored at –80°C. We humanely euthanized hamsters that did not develop clinical signs at 550 days after treatment and collected brains. We further confirmed prion disease by using biochemical analyses. All animal experimentation was performed following National Institutes of Health guidelines (*4*) and approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston.

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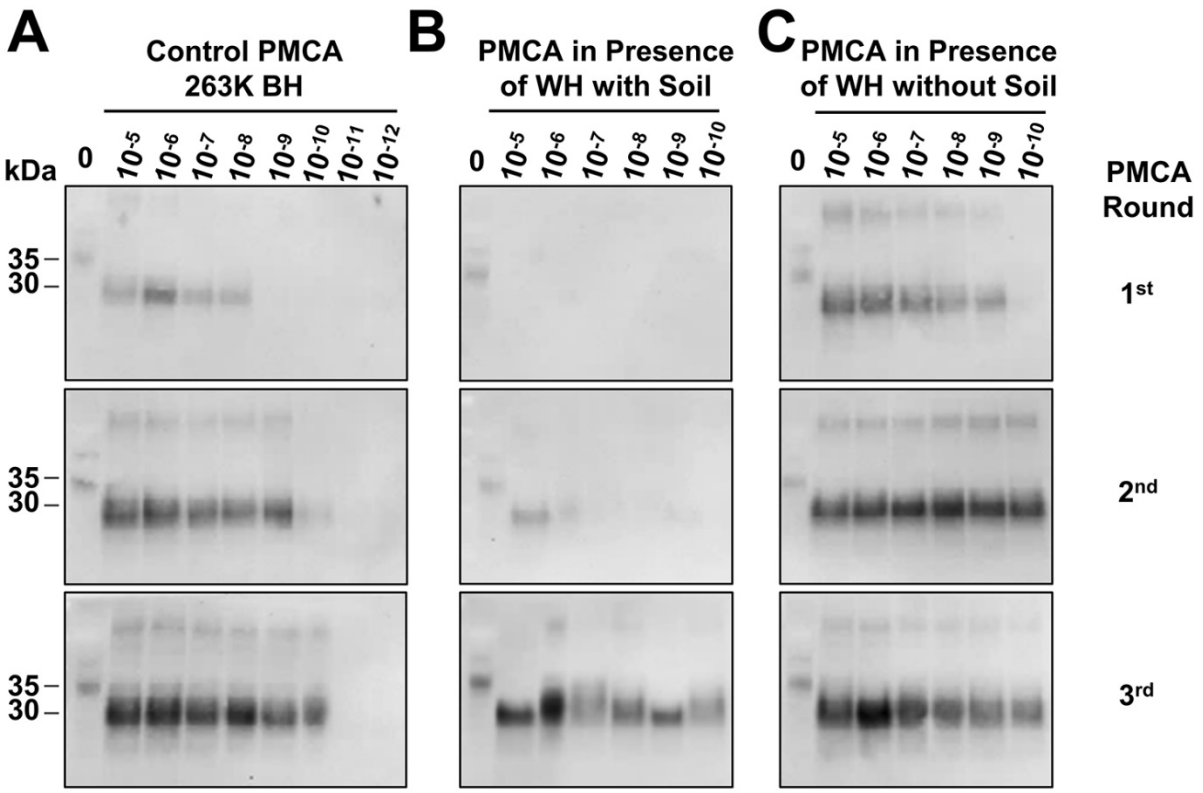
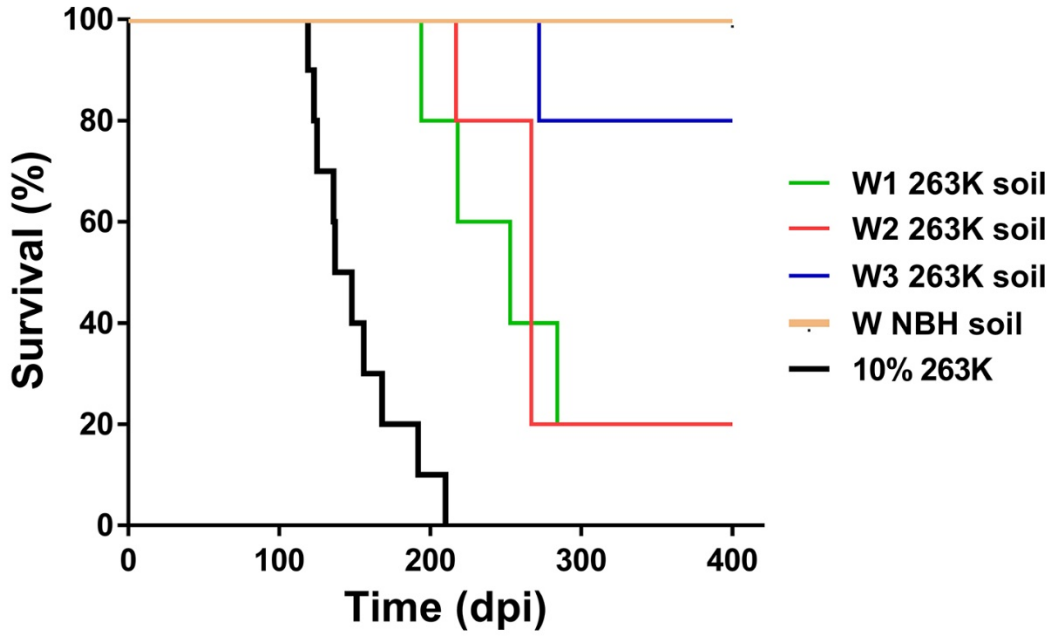


Appendix Figure 1. Results of dilution experiment using 263K hamster brain homogenate (BH) with and without worm homogenate (WH) to determine putative interference of worm and soil inside the animal body on the PMCA reaction. Samples were subjected to 3 serial rounds of 96 PMCA cycles and PrP^{Sc} signal was detected by Western blot after proteinase K (PK) digestion. For these studies, we used the same 263K BH as inoculum and wild type hamster BH as substrate. All samples were digested in PK except the normal brain homogenate (NBH) used as a migration control (lane 0). A) Control PMCA for amplification of PrP^{Sc} in 263K BH in the absence of WH. B) PMCA in the presence of 10% total WH without removing soil from inside worms before creating homogenate. C) PMCA in soil-devoid WH. No signal was ever detected in the absence of 263K BH (data not shown). For each reaction, lane 0 indicates NBH control and other lanes indicate homogenate dilution. Numbers on the left indicate molecular weight markers. Numbers on the right indicate PMCA round. PMCA, protein misfolding cyclic amplification.



Appendix Figure 2. Survival curves for hamsters injected with homogenate from worms exposed to prion contaminated soil. Groups of 5 naive hamsters received intraperitoneal injection of 600 µL of 10% wt/vol WH derived from 3 different worms (W1, W2, W3). For a negative control (yellow line), 5 hamsters received intraperitoneal injection of 600 µL of a 10% wt/vol homogenate from a worm exposed to soil mixed with PrP^{Sc}–free brain extract. For a positive control (black line), 10 hamsters received intraperitoneal injection of 100 µL of 10% wt/vol brain homogenate from a 263K sick animal. Diseased animals exhibited typical 263K clinical signs, including ataxia, hyperactivity, aggression, and sensitivity to noise. Hamsters not showing signs of prion disease were humanely euthanized at 550 dpi. dpi, days post infection; NBH, normal brain homogenate; PrP^{Sc}, prion protein; WH, worm homogenate.